Appl Microbiol Biotechnol (2000) 53: 583–590

ORIGINAL PAPER

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Genes encoding cytochrome P450 and monooxygenase enzymes define one end of the aflatoxin pathway gene cluster in *Aspergillus parasiticus*

Received: 21 May 1999 / Received revision: 26 October 1999 / Accepted: 12 November 1999

Abstract The identification of overlapping cosmids resulted in the discovery of the aflatoxin biosynthetic pathway gene cluster in *Aspergillus flavus* and *A. parasiticus*. This finding led to the cloning and characterization of one regulatory and 16 structural genes involved in aflatoxin biosynthesis, including the most recent report on the gene, *ordA,* which has been identified to be involved in the formation of four aflatoxins (B_1, B_2, G_1 and G_2). However, these genes do not account for all the identified chemical/biochemical steps in aflatoxin synthesis and efforts are underway to identify the genes controlling the other steps. We are also attempting to define the outer boundaries of the aflatoxin pathway gene cluster in the *Aspergillus* genome. For this goal, we extended sequencing in both directions from the existing (60 kb) aflatoxin pathway gene cluster, beyond the *pksA* gene at one end and the *omtA* gene at the other. Within the 25-kb genomic DNA sequence determined at the *omtA* end of the cluster, several new gene sequences were identified. The recently reported genes, *vbs* and *ordA*, were found within this 25-kb region. Two additional genes were also found in this region, a cytochrome P450 monooxygenase encoding gene, tentatively named *cypX*, and a monooxygenase encoding gene, tentatively named *moxY*, and these are also reported in this study. The sequence beyond these genes showed a 5-kb non-coding region of DNA followed by the presence of a cluster of genes probably involved in sugar metabolism. Northern blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) studies demonstrated that the genes, *cypX* and *moxY*, are expressed concurrently with genes involved in aflatoxin biosynthesis. Therefore, the two putative aflatoxin pathway genes *cypX* and *moxY* followed by a 5-kb non-coding region of DNA define one end of the boundary of the aflatoxin pathway gene cluster in *A. parasiticus*.

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Introduction

Aflatoxins are carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus* in various crops and commodities, both pre- and post-harvest. Contamination of agricultural commodities with aflatoxins results in economic and food safety problems worldwide (Bhatnagar et al. 1997; Cleveland and Bhatnagar 1992; Jelinek et al. 1989). Aflatoxin biosynthesis in the toxigenic fungi consists of multi-enzyme reactions starting from the synthesis of polyketides. At least 18 enzymatic reactions have been characterized or proposed (Cleveland et al. 1997; Minto and Townsend 1997; Payne and Brown 1998). Most of the corresponding genes have been isolated and characterized (*aflJ*, Meyers et al. 1998; *aflR*, Chang et al. 1995b; Payne et al. 1993; Woloshuk et al. 1994; *avnA*, Yu et al. 1997; *fas-1* (*fas-1A*), Mahanti et al. 1996; *nor-1*, Trail et al. 1994; *norA*, Cary et al. 1996;; *omtA*, Yu et al. 1993; *ordA*, Yu et al. 1998; *pksA*, Chang et al. 1995a; *vbs*, Silva et al. 1996; *ver-1*, Skory et al. 1992). So far, a total of 22 genes or open reading frames (ORFs) have been identified as a cluster of genes within one 60-kb region of the *A. parasiticus* genome (Cleveland et al. 1997; Yu et al. 1995) and 25 co-regulated transcripts within a 60-kb DNA region for sterigmatocystin biosynthesis in *A. nidulans* (Brown et al. 1996).

However, the genes in the cluster whose functions have already been determined in *A. parasiticus* and *A. flavus* cannot account for all the enzymatic steps in the aflatoxin biosynthetic pathway. In order to identify other potential aflatoxin pathway genes and to establish a complete aflatoxin pathway gene cluster, we are sequencing and analyzing DNA at both ends of the identified cluster, marked by the *pksA* gene at one end and the *omtA* gene at the other. Here we report the

analysis of an additional 25-kb DNA region beyond *omtA* at one end of the aflatoxin pathway gene cluster. Two characterized genes, *vbs* (Silva et al. 1996) and either *ordA* in *A. parasiticus* (Yu, et al. 1998), or *ord1* in *A. flavus* (Prieto and Woloshuk 1997), and two additional ORFs, *cypX* and *moxY* [previously named *aflB* and *aflW* (Woloshuk and Prieto 1998)], were found to be located within this region (Fig. 1). It defines one end of this gene cluster and reveals a sequence of two genes putatively encoding cytochrome P450 monooxygenase and monooxygenase. The *cypX* and *moxY* are homologous to *stcB* and *stcW,* respectively, in the *A. nidulans* sterigmatocystin pathway gene cluster. We have also demonstrated that these genes appear to be involved in aflatoxin biosynthesis. A sugar metabolism gene cluster is located 5 kb from the *moxY* gene. Therefore, the *moxY* gene defines one end of the aflatoxin pathway gene cluster in the *omtA* orientation in *A. parasiticus* and the 5-kb non-coding sequence marks the boundary of the aflatoxin pathway gene cluster.

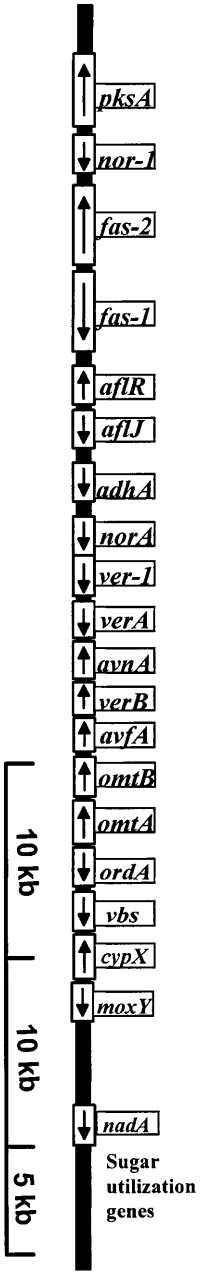

Fig. 1 The extended aflatoxin pathway gene cluster. In this schematic representation of the aflatoxin biosynthesis pathway gene cluster, the genes are represented by *boxes* on the *vertical bar*. Their names are given in the *labels* on the side of each gene. *Arrows* inside the boxes indicate the direction of transcription

Materials and methods

Fungal and bacterial strains and culture conditions

The fungal strain *A. parasiticus* SRRC 143 (ATCC 56775) produces aflatoxins B_1, B_2, G_1, and G_2; and *A. flavus* strain SRRC 1007 produces aflatoxins B_1 and B_2. Both strains were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich.). PDA was also used for detecting aflatoxin production. A&M medium (Adye and Mateles 1964) was used for growing fungal mycelia as submerged cultures. *A. parasiticus* wild-type strain SRRC 143 was grown for 48 h in A&M medium with constant shaking at 150 rpm at 29 °C. The resulting mycelia were harvested and pulverized to a fine powder in the presence of liquid nitrogen, using a Waring blender. *Escherichia coli* strain DH5a was used to amplify and maintain cosmid clones and plasmid subclones.

Subcloning and sequencing

The *A. parasiticus* genomic DNA cosmid library was constructed previously (Skory et al. 1992). The genomic cosmid clone no. 2 contains *omtA* and harbors additional aflatoxin pathway genes. A 9-kb *Sal*I/*Xba*I DNA fragment and a 3-kb *Sac*II DNA fragment adjacent to the *omtA* gene were subcloned into a pBC vector (Stratagene, La Jolla, Calif.). Both strands of the subclones were sequenced initially using universal primers by primer walking strategy. The additional sequence was determined by direct sequencing of the cosmid clone no. 2. The sequencing procedures and the primers generated were as reported earlier (Yu et al. 1997).

Isolation of mRNA and reverse transcriptase-polymerase chain reaction

A. parasiticus wild-type strain SRRC 143 and conducive *A. flavus* strain SRRC 1007 were grown in non-aflatoxin conducive medium, the peptone–mineral salt (PMS) medium (Adye and Mateles, 1964), for 48 h. The mycelia were transferred to aflatoxin-supportive medium, the glucose–mineral salt (GMS) medium in which peptone was replaced by glucose (Adye and Mateles 1964). The resulting mycelia were harvested at 24 h and 48 h after transfer from PMS to GMS and pulverized to a fine powder in the presence of liquid nitrogen, in a Waring blender. Total RNA was isolated from the mycelia by the column purification method, using RNeasy Plant Mini Kit (Qiagen, Valencia, Calif.). Poly-A mRNA was purified from the total RNA with a PolyATtrack mRNA isolation system (Promega, Madison, Wis.), according to the instruction manual. First strand cDNA was synthesized by Advantage reverse transcriptase-for-polymerase chain reaction (RT-for-PCR) Kit (Clontech, Palo Alto, Calif.) and used as the template in the subsequent PCR reactions. The PCR amplification was 30 cycles of: 94 °C for 45 s denaturing, 60 °C for 45 s annealing, and 72 °C for 2 min extending.

Nucleotide sequence accession number

The genomic DNA nucleotide sequence from *omtA* up to *nadA*, including the spacer region reported here, has been submitted to

GenBank under accession number AF 169016. Only the *cypX* and *moxY* portion of the DNA sequences are presented in this paper.

Results

Localization and identification of aflatoxin pathway genes in *A. parasiticus*

We have determined the sequence of over 25 kb genomic DNA, spanning the *omtA* and *nadA* genes (Fig. 1). The linear relationship of three reported genes, *omtA*, *ordA*, and *vbs* and the two newly identified genes has been determined (Fig. 1). The *ordA* gene was located between the *omtA* and *vbs* genes. The *ordA* gene was transcribed in a direction opposite to *omtA* with an intergenic region of 1320 bp from their translational start sites; but in the direction of the *vbs* gene with a 511-bp spacer from the *ordA* translational stop to the *vbs* gene translational start site (Fig. 1). Next to *vbs*, two additional genes were identified which showed high homology to cytochrome P450 monooxygenases (over 60%) and monooxygenases (over 50%), respectively, and were tentatively named *cypX* and *moxY* (Fig. 1). The *cypX* and *moxY* are divergently transcribed with an intergenic region of 620 bp (Fig. 1).

The *cypX* and *moxY* genes define the end of the aflatoxin pathway gene cluster in the *omtA* direction in *A. parasiticus*

The genes involved in aflatoxin biosynthesis are clustered and efficiently compacted within a 60-kb fragment in the genome. So far all of the identified genes and ORFs in the aflatoxin pathway gene cluster are functionally involved in aflatoxin biosynthesis, either as structural genes such as *pksA*, *nor-1*, *ver-1*, *avnA*, *omtA*, *ordA*, or as a regulatory gene (*aflR*). No pseudo-gene has been found within the gene cluster. The distance between each of these genes is no more than 1.5 kb with an average of around 0.8 kb. Within the 25-kb sequence, beyond *ordA* gene at one end of the aflatoxin pathway gene cluster, there is a 5-kb region following the *moxY* gene where no ORF was found. The *cypX* and *moxY* are functional genes (as discussed later) and therefore part of the aflatoxin pathway gene cluster, as opposed to this 5-kb non-coding region. Preliminary results indicate that several ORFs can be identified beyond the 5-kb non-coding region. A gene, tentatively named *nadA*, was found immediately after the 5-kb region (Fig. 1). The *nadA* gene showed homology with a NADH oxidase-encoding gene which may be involved in the breakdown of hexose to triose in glycolysis (unpublished observations). There is a potential sugar utilization gene cluster, including genes coding for a hexose transporter protein, glucosidase, and a sugar regulatory gene, located immediately next to the *nadA* gene (data not shown). The *nadA* gene may be a member of a putative sugar utilization gene cluster. Thus, this 5-kb open stretch of DNA apparently serves as a spacer between the two clusters. No aflatoxin pathway-related gene was identified beyond the *moxY* gene. Therefore, the *cypX* and *moxY* genes may well define the end of the aflatoxin pathway gene cluster in the *ordA* direction.

cypX and *moxY* are expressed under aflatoxin-conducive conditions

Aflatoxin pathway genes are known to be expressed only under conducive conditions such as those present in GMS medium but not in PMS medium. Transcript detection by RT-PCR showed that *cypX* and *moxY* were expressed only under aflatoxin-conducive conditions (Fig. 2). RT-PCR of the *cypX* and *moxY* genes from mycelia grown in GMS medium for 24 h and 48 h produced cDNA bands of 1.568 kb (Fig. 2A, lanes 3 and 4) and 1.471 kb (Fig. 2B, lanes 3 and 4), respectively. No cDNA band of either *cypX* (Fig. 2A, lane 2) or *moxY* (Fig. 2B, lane 2) was amplified from mycelia grown in PMS medium. As a positive control, the *nmt1* gene (Cary and Bhatnagar 1995), a constitutively expressed thiamine biosynthetic pathway gene, was included. The results demonstrated that a 1.061 kb *nmt1* mRNA was detected from mycelia grown both in PMS and in GMS media (Fig. 2C, lanes 2, 3 and 4). Northern blot analysis is consistent with the RT-PCR results (data not shown).

cypX codes for a cytochrome P450 monooxygenase

The *cypX* gene contains a coding sequence of 1473 bp and is capable of encoding a protein consisting of 508 amino acid residues with a calculated molecular mass of 56.3 kDa. The deduced gene product contains the three highly conserved motifs characteristic of all cytochrome P450 type enzymes including monooxygenases, reductases and dehydrogenases (Bozak et al. 1990; Maloney and VanEtten 1994; Porter and Coon 1991; Van den Brink et al. 1998). These motifs are located within the carboxy terminal part of the protein and are believed to be the active sites of the P450 enzymes (Nhamburo et al. 1989; Porter and Coon 1991; Potenza et al. 1989; Prieto and Woloshuk 1997; Van den Brink et al. 1998; Yu et al. 1997, 1998). The conserved amino acid residue "F – – G – – – C – G" is the major motif. The cysteine residue within this motif provides a ligand for heme-binding (Nhamburo et al. 1989; Prieto and Woloshuk 1997; Yu et al. 1997, 1998). The "E – – R" sequence is believed to be in the K-helix for hydrogen bonding with the neighboring sequence. The "A – - – T" sequence is thought to encode the I-helix oxygen-binding pocket.

At the genomic DNA level no significant sequence homology (overall about 40%) was found between the *cypX* and *stcB* genes of the *A. nidulans* sterigmatocystin biosynthetic pathway gene cluster (Brown et al. 1996). However, the deduced amino acid sequence of *cypX* showed significant identity (67.4%) to that of the iden-

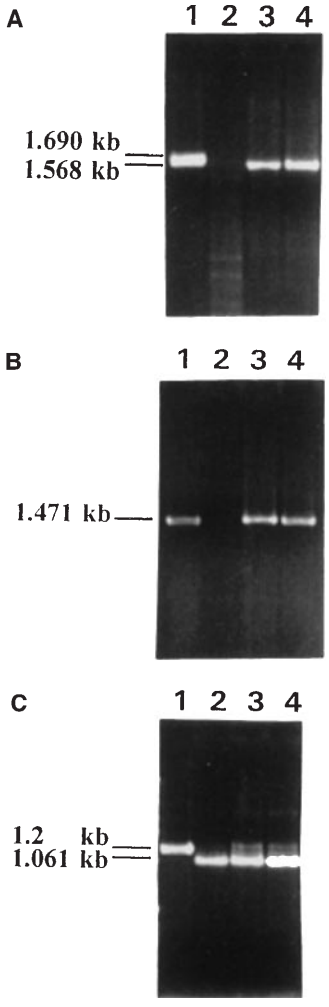

Fig. 2A–C Reverse transcriptase-polymerase chain reaction (RT-PCR) of *cypX* and *moxY*. RT-PCR was performed to detect mRNAs of *Aspergillus parasiticus* from mycelia grown in glucose–mineral salt (GMS) and peptone–mineral salt (PMS) media for *cypX* (**A**) and *moxY* (**B**) using primers outside the putative translational start and stop codons. The constitutively expressed gene, *nmt1* (**C**) in the thiamine biosynthetic pathway, was included as a positive control. *Lane 1* Genomic DNA as template, *lane 2* RNAs purified from mycelia grown in PMS medium for 48 h as template, *lane 3* RNAs purified from mycelia grown in GMS medium for 24 h as template, *lane 4* RNAs purified from mycelia grown in GMS medium for 48 h as template. PCR products were separated in 2% agarose gel under 80 V for 90 min

tified gene, *stcB*, which encodes a protein consisting of 435 amino acid residues in *A. nidulans*. In the promoter region of the *cypX* gene a typical aflatoxin pathway regulatory protein (AFLR) binding motif, the canonical $TCGN_5CGA$ binding sequence (Ehrlich et al. 1999), was located at the –461 position. Several possible TATA boxes were also identified (Fig. 3, underlined). Based on the sequence alignment, RT-PCR data and the cDNA sequence, two introns of 67 and 55 bp each have been identified in the *cypX* gene, separating the three exons in *A. parasiticus*. However its homolog in *A. nidulans*, the *stcB* gene, contains three introns of 41, 146, and 71 bp each, respectively. The intervening sequences of *stcB* were not only different in length from those of *cypX* but also spliced out at different locations.

moxY codes for a monooxygenase

moxY consists of a coding sequence of 1446 bp and is capable of encoding a protein of 481 amino acids with a calculated molecular mass of 55 kDa. In the promoter region, the typical AFLR binding motif was located at the –170 position. Several possible TATA boxes were also identified (Fig. 4, underlined). The BLAST search revealed that the deduced gene product showed high homologies (over 50%) with monooxygenases in the GenBank database (data not shown). However, no characteristic motif was apparent (Fig. 4) such as those found in cytochrome P450 type monooxygenases. At the genomic DNA level no significant sequence homology (about 40%) was found between the *moxY* and *stcW* genes. However, the deduced amino acid sequence showed significant identity (69%) to the 488 amino acids encoded by the *stcW*gene in *A. nidulans* (Brown et al. 1996). The *stcW* gene contains three introns of 48, 54, and 64 bp each respectively in *A. nidulans*. However, no intron was found within *moxY* in *A. parasiticus*.

Discussion

By identifying the 5-kb open stretch of the spacer DNA surrounded by the potential sugar utilization gene cluster at one end and the two putative genes *cypX* and *moxY* of the aflatoxin biosynthesis pathway at the other end, we have concluded that the genes *cypX* and *moxY* define the boundary at one end of the *A. parasiticus* aflatoxin pathway gene cluster in the *omtA* orientation (Fig. 1).

Cytochrome P450 type enzymes have been identified to be involved in several mycotoxin biosynthetic pathways. The genes for these enzymes were found within the aflatoxin (Prieto and Woloshuk 1997; Yu et al. 1997, 1998), sterigmatocystin (Brown et al. 1996), and trichothecene (Hohn et al. 1995) biosynthetic pathway gene clusters. We have reported earlier that *avnA* (Yu et al. 1997) and *ordA* (Yu et al. 1998) located in the *A. parasiticus* and *A. flavus* aflatoxin pathway gene cluster were found to be cytochrome P450 type monooxygenases. The *cypX* gene product is another example of a cytochrome P450 type enzyme involved in aflatoxin biosynthesis.

Based on amino acid homology, the functions of both *cypX* and *moxY* gene products would be to carry out oxidation or reduction reactions involved in the modification of aflatoxin precursors in aflatoxin biosynthesis (Bhatnagar et al. 1992). The exact function of the two genes, *cypX* and *moxY*, in aflatoxin biosynthesis is being determined by gene inactivation (knockout) experiments. Disruption of the homolog genes, *stcB* and *stcW*, in *A. nidulans* has not yet provided conclusive results as to the function of these two genes (Keller, personal communication).

The degree of homology between the counterpart genes in *A. parasiticus* and *A. nidulans* in general varies between 40–70%. The DNA sequences of the genes identified in this study also demonstrated a similar homology (about 40%), i.e. between *cypX* and *stcB*, and between *moxY* and *stcW*. However, the protein

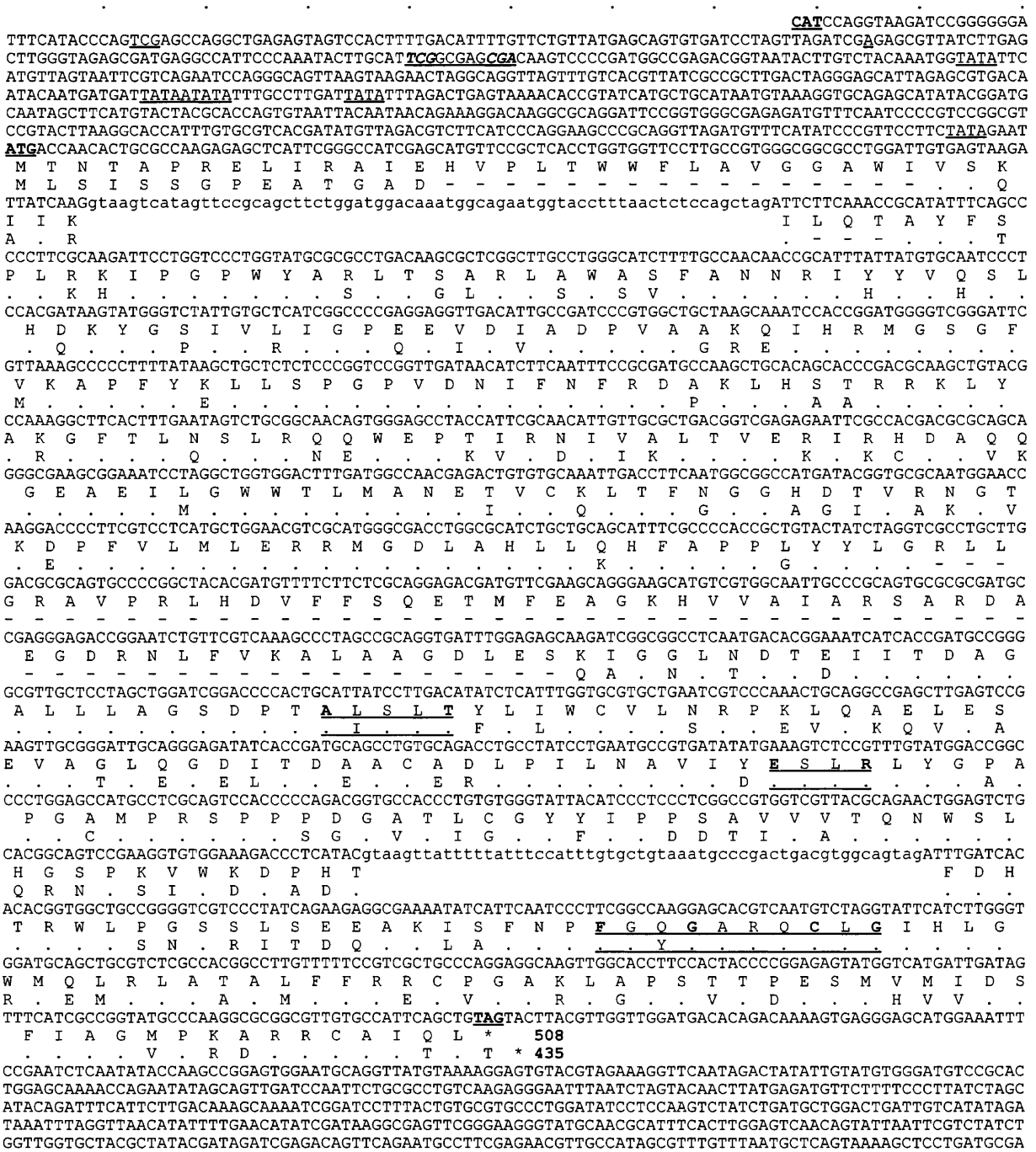

Fig. 3 Genomic DNA sequence and comparison of the deduced amino acid sequence of the *cypX* in *A. parasiticus* with *stcB* in *A. nidulans*. The *cypX* DNA sequence from *A. parasiticus* SRRC 143 is presented, including the promoter region up to the *moxY* translational start site. The translation start codon (ATG) and the translation termination codon (TAG) are *underlined* and printed in *bold*. In the promoter region the *aflR* binding consensus sequence, the canonical $TCGN_5CGA$ binding site, is printed in *bold*, *italicized*, and *underlined*. The putative TATA sequences are *underlined* as well. The putative intron sequences are denoted by *lowercase letters*. The deduced amino acid sequence of *cypX* gene product is presented under the corresponding codons and aligned with that of the sterigmatocystin pathway gene *stcB* in *A. nidulans*. The identical amino acids are represented by *dots* and gaps are represented by *hyphens*. The highly conserved motifs of the P450 enzyme are *underlined* and conserved amino acid residues are printed in *bold*. *Numbers* on the left give the coordinates, both of nucleotides starting from the ATG start codon and of amino acids starting from the initiator methionine

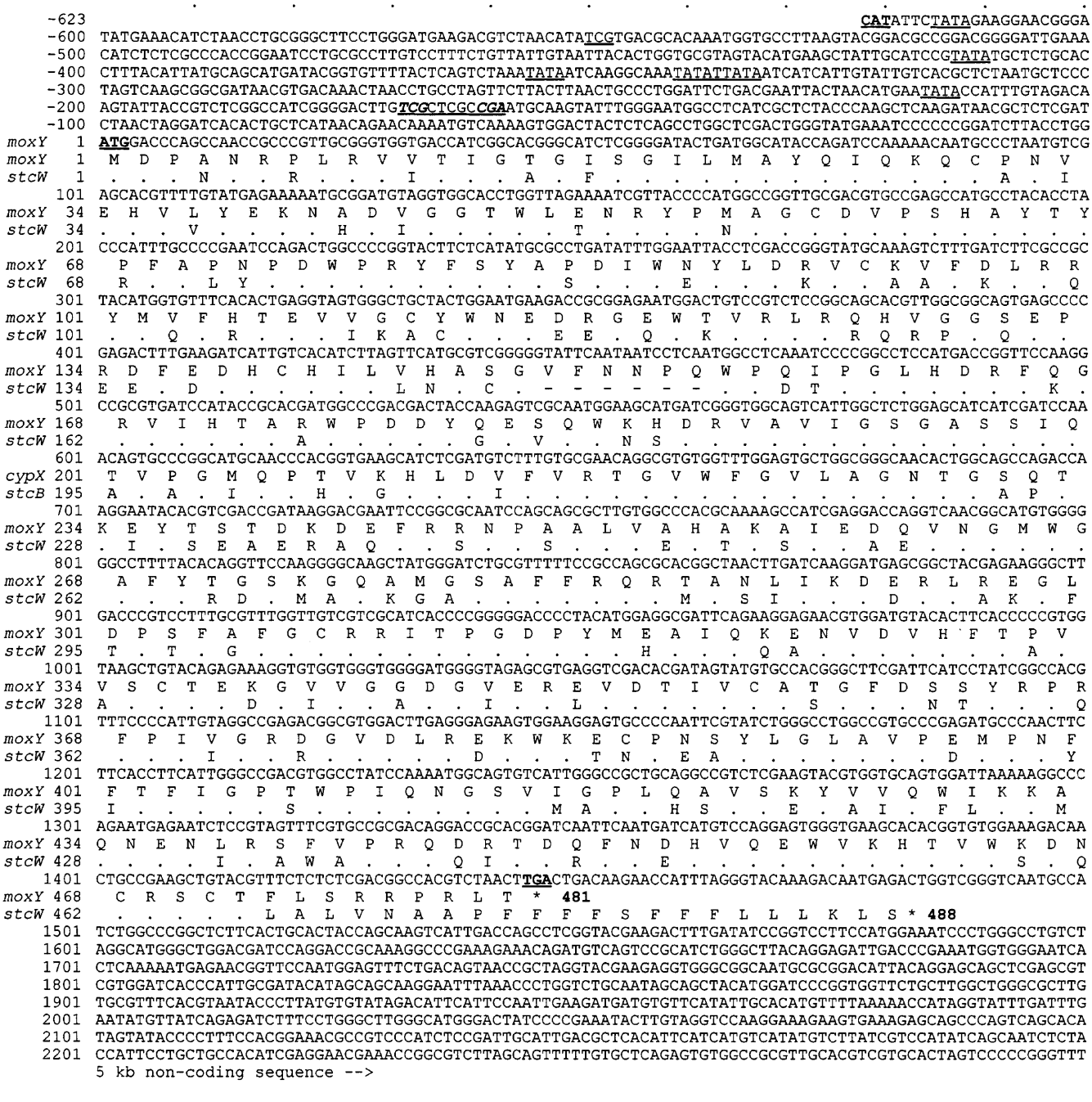

Fig. 4 Genomic DNA sequence and comparison of the deduced amino acid sequence of *moxY* in *A. parasiticus* with *stcW* in *A. nidulans*. The *moxY* DNA sequence from *A. parasiticus* SRRC 143 is presented, including the promoter region up to the *cypX* translational start site. The translation start codon (ATG) and the translation termination codon (TAG) are *underlined* and printed in *bold*. In the promoter region the *aflR* binding consensus sequence, the canonical $TCGN_5CGA$ binding site, is printed in *bold*, *italicized*, and *underlined*. The putative TATA sequences are *underlined* as well. The putative intron sequences are denoted by *lowercase letters*. The deduced amino acid sequence of *moxY* is presented under the corresponding codons and aligned with that of the sterigmatocystin pathway gene *stcW* in *A. nidulans*. Identical amino acids are represented with *dots* and gaps are presented by *hyphens*. *Numbers* on the left give the coordinates, both of nucleotides starting from the ATG start codon and of amino acids starting from the initiator methionine

sequences encoded by these genes are significantly conserved (67–69%, this study). The functionally indispensable motifs such as the typical cytochrome P450 motifs, are very significantly conserved (>99%) at the amino acid level between the counterpart genes.

Generally, the introns in fungal systems are around 40–70 bp in length (Yu et al. 1997, 1998). Careful comparison showed that the amino acid sequence deduced from at least part of the second intron of *stcB* has over 60% homology with that of the *cypX* coding sequence. The *stcB* in *A. nidulans* may encode a polypeptide longer than 435 amino acids if the reported 146-bp second intron actually contains part of the coding sequence.

The genes of the aflatoxin biosynthetic pathway in *A. flavus* and *A. parasiticus* are located in an identical order on the chromosomes of the two organisms (Cleveland et al. 1997; Yu et al. 1995). But the organization of the identified counterpart genes of the aflatoxin pathway gene cluster in both *A. flavus* and *A. parasiticus* is quite different from the sterigmatocystin pathway gene cluster in *A. nidulans*. The *stcB* and *stcW*, which are surrounded by *stcA* and *stcC*, and *stcV* and *stcX* respectively, do not define either end of the sterigmatocystin pathway gene cluster, whereas *cypX* and *moxY* define one end of the aflatoxin pathway gene cluster in *A. parasiticus*.

Acknowledgements We thank Karen Gillespie for technical assistance and Janell Becker for secretarial help.

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Note added in proof The function of *stcB* and *stcW* has been proposed to be involved in the conversion process from averufin to verciconal hemiacetal acetate. Keller NP, Watanabe CMH, Kelkar HS, Adams TH, Townsend CA (2000) Requirement of monooxygenase-mediated steps for sterigmatocystin biosynthesis by *Aspergillus nidulans*. Appl Environ Microbiol 66: 359–362